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Environmental Novelty Activates β_2-Adrenergic Signaling to Prevent the Impairment of Hippocampal LTP by Aβ Oligomers

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SUMMARY

A central question about human brain aging is whether cognitive enrichment slows the development of Alzheimer changes. Here, we show that prolonged exposure to an enriched environment (EE) facilitated signaling in the hippocampus of wild-type mice that promoted long-term potentiation. A key feature of the EE effect was activation of β_2-adrenergic receptors and downstream cAMP/PKA signaling. This EE pathway prevented LTP inhibition by soluble oligomers of amyloid β-protein (Aβ) isolated from AD cortex. Protection by EE occurred in both young and middle-aged wild-type mice. Exposure to novelty afforded greater protection than did aerobic exercise. Mice chronically fed a β-adrenergic agonist without EE were protected from hippocampal impairment by Aβ oligomers. Thus, EE enhances hippocampal synaptic plasticity by activating β-adrenoceptor signaling and mitigating synaptotoxicity of human Aβ oligomers. These mechanistic insights support using prolonged exposure to cognitive novelty and/or oral β-adrenergic agonists to lessen the effects of Aβ accumulation during aging.

INTRODUCTION

Rodents living in a simple laboratory environment access only food and water. Adding multiple novel objects and running wheels to their cages, referred to as environmental enrichment (EE), has been shown in many studies to improve memory and its neuroanatomical and biochemical substrates. Humans who develop AD invariably accumulate Aβ in limbic and association cortices and undergo an insidious erosion of memory and cognition. Mice transgenic (tg) for human APP (the precursor of Aβ) that are exposed to EE generally show an improvement in cognitive deficits compared to tg mice in standard housing (SH) (Arendash et al., 2004; Jankowsky et al., 2005; Wolf et al., 2006; Berardi et al., 2007; Costa et al., 2007; Valero et al., 2011; see Table S1 available online). However, these studies perforce include EE effects on the processing of markedly overexpressed mutant APP in models of rare genetic forms of AD, making it impossible to separate the neuroprotective effects of EE from effects on the profound Aβ pathology such animals have. The vast majority of AD cases suffer the late-onset, largely "sporadic" form of the disease, whereas a very small number of familial cases are caused by deterministic genetic mutations. These and other data suggest that environmental factors may play a role in the development of many cases of typical (late-onset) AD. Experimentally, soluble Aβ oligomers, including those isolated directly from AD brain tissue, have been shown to potently block hippocampal long-term potentiation (LTP), an electrophysiological correlate of learning and memory, whereas insoluble amyloid plaque cores have far less bioactivity (Selkoe 2002; Walsh et al., 2002; Shankar et al., 2008; Wilcox et al., 2011). The negative effects of Aβ oligomers on hippocampal LTP provide a widely validated experimental system for deciphering some of the mechanisms of early AD pathogenesis (Nalbantoglu et al., 1997; Klyubin et al., 2011).

While a few studies have examined the effects of EE on APP processing and Aβ economy in tg mice strongly overexpressing human APP (Table S1), we are unaware of reports on whether EE can alter the vulnerability of wild-type adult neurons to the synaptic effects of soluble Aβ oligomers, which are believed to mediate neurotoxicity in AD (Selkoe 2002). Studying the effects of EE in wild-type animals exposed to human Aβ oligomers can better model the early development of Aβ-mediated neurotoxicity in the majority of humans without a deterministic genetic predisposition to AD. It is in such patients that chronic environmental factors are likely to play an important role in AD pathogenesis. Here, we report that activation of β-ARs by exposing normal mice, including mature adults, to 2 months of environmental novelty fully prevents the impairment of hippocampal synaptic plasticity by Aβ oligomers, and this protection can be mimicked by prolonged feeding of a β-adrenergic agonist without EE.

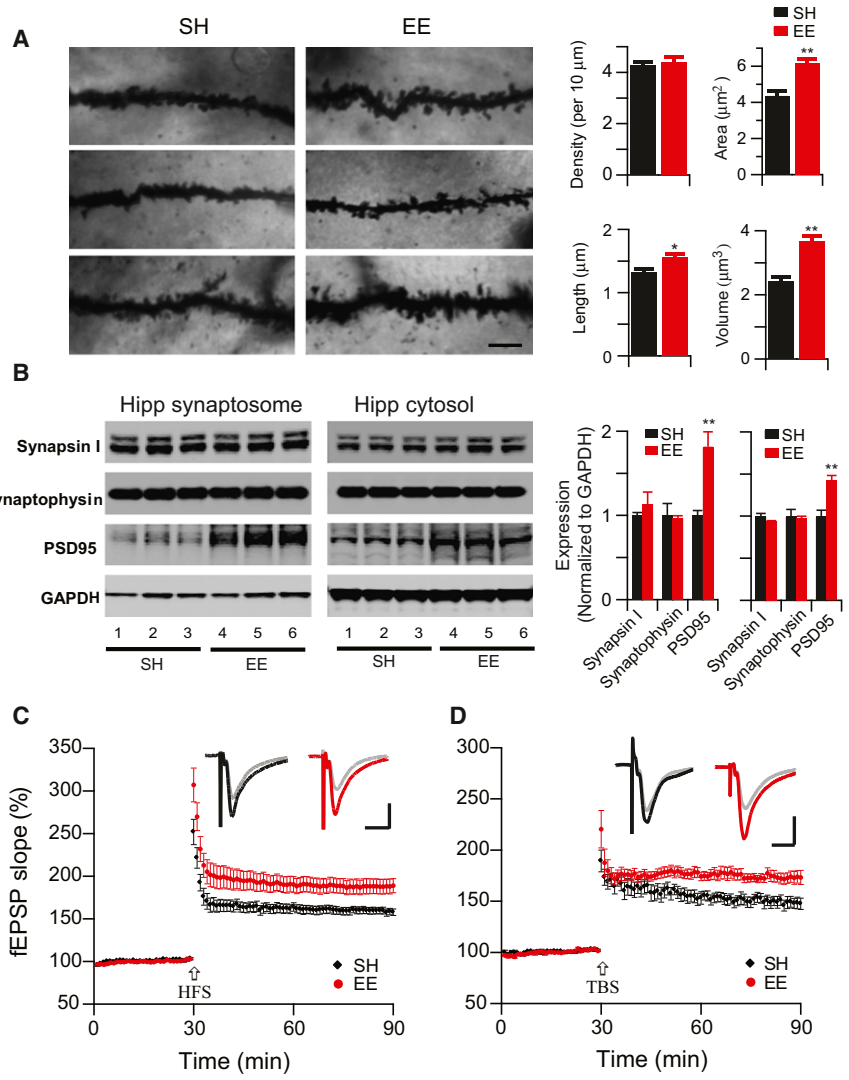

Figure 1. Prolonged Exposure to an Enriched Environment (EE) Enlarges Dendritic Spines and Enhances Hippocampal Long-Term Potentiation

(A) Representative Golgi stains and quantification: hippocampal dendritic spine area, length and volume, but not density, were significantly increased by EE training. Scale bar, 5 μm. (*$p < 0.05$; **$p < 0.01$).

(B) The presynaptic proteins, synapsin I and synaptophysin, were unchanged in response to EE, while the postsynaptic protein, PSD95, rose significantly (**$p < 0.01$) in both synaptosome and cytosol fractions of hippocampus.

(C) EE significantly increased LTP induced by high frequency stimulation (HFS, arrow) in the CA1 region of hippocampal slices (red circles, n = 28 slices/from 25 mice) versus those of mice in standard housing (SH) (black diamonds, n = 13/13).

(D) EE significantly increased LTP induced by theta-burst stimulation (TBS, arrow) of hippocampal slices (red circles, n = 15/12) versus those in SH (black diamonds, n = 11/10). Data are means ± SEM. Inset traces are typical field excitatory postsynaptic potentials (fEPSPs) recorded before (gray) and after (black or red) HFS or TBS. Horizontal bars: 10 ms; vertical bars: 0.5 mV.

See also Figure S1.

RESULTS

EE Enlarges Dendritic Spines and Enhances Hippocampal LTP

We first investigated the potential benefits of exposure to EE on the synaptotoxicity of soluble Aβ oligomers by initiating EE training at age 14 days and continuing daily for 4 weeks, because EE exposure during postnatal development is known to have greater benefits than in adult mice (Escorihuela et al., 1994; Cancedda et al., 2004; Li et al., 2006). Six to eight outbred wild-type (WT) mice (C57Bl/6 × 129) were housed together for 8 hr per day 7 days per week in a large cage (38 × 60 cm) having several different brightly colored mouse toys and a running wheel (see Experimental Procedures). To encourage active exploration of a novel environment, new toys were swapped for existing ones every day. The activity of the individual mice (exploring the objects and/or running on a wheel) was monitored ~2–4 times during each 8 hr EE training session; a very few animals that persistently showed no interest in exploratory or running activity were removed from the EE groups. Control littermate mice were housed in the same room in standard cages with only bedding and access to water and food pellets (SH).

To verify that the neural effects of our EE protocol were generally consistent with those in prior studies, we assessed morphological and biochemical changes in the hippocampus after the 4 week EE exposure using Golgi impregnation, BrdU staining, quantitative western blotting (WB) of synaptic proteins, and electrophysiology. Golgi staining showed that EE training significantly increased the length and area of dendritic spines of pyramidal neurons in CA1 of hippocampus, resulting in a mean increase in spine volume of 151% compared to SH littermates, i.e., from 2.42 μm^3 to 3.66 μm^3 (Figure 1A), while astrocyte numbers did not appear to change (Figure S1A). Next, we isolated the synaptic membrane-enriched fraction (synaptosomes) and the high-speed cytosol fraction from both hippocampus and cerebral cortex of EE and SH mice and performed quantitative WB for the presynaptic proteins synapsin I and synaptophysin; no significant differences were found (Figures 1B and S1C). Electrophysiological recordings were consistent with the latter finding, as paired-pulse facilitation, a measure of presynaptic efficacy, was at similar levels in hippocampal slices of mice with versus without EE exposure (Figure S1B). In contrast, levels of the postsynaptic protein PSD95 rose ~80% in hippocampus in the EE mice (Figure 1B); there was no significant change in the cerebral cortex

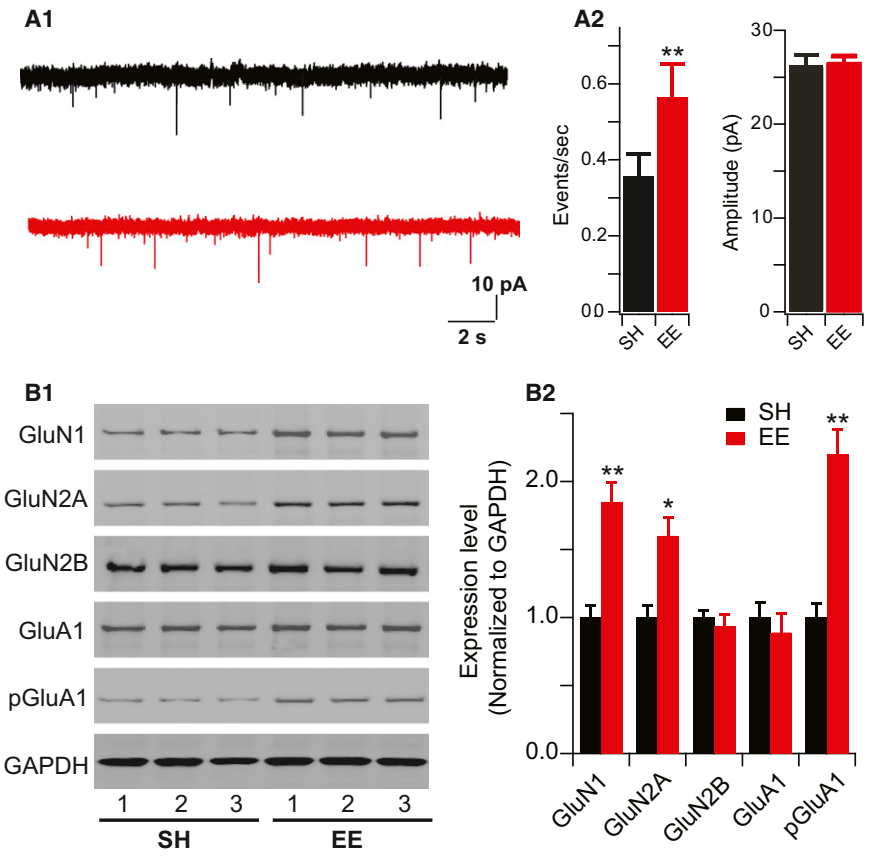

Figure 2. EE Increases the Frequency of Miniature Excitatory Postsynaptic Currents (mEPSC) in CA1 Neurons and the Phosphorylation of AMPAR Subunit GluA1 at S845

(A) Representative voltage-clamp recordings (A1) from pyramidal neurons in SH (black) and EE mice (red) and summary data (A2).

(B) Neuronal receptors implicated in synaptic plasticity and memory (GluN1, GluN2A, GluN2B, GluA1, and pGluA1) were analyzed by quantitative WB after 4 week EE training. Representative blots show the protein levels from hippocampal synaptosomes (B1). Summary data (bars) are shown on (B2).

Error bars are ±SEM (*$p < 0.05$; **$p < 0.01$). See also Figure S2.

(Figure S1C). Hippocampal LTP, a sensitive and readily quantifiable electrophysiological correlate of synaptic plasticity, was significantly increased by EE, as shown by the magnitude of LTP induced by either a high-frequency stimulus (HFS) or theta-burst stimulation (TBS) versus that in SH mice (HFS: 187% ± 9%, n = 28 slices/from 25 mice, versus 158% ± 4%, n = 13/13; TBS: 167% ± 5%, n = 15/12, versus 148% ± 6%, n = 11/10; $p < 0.05$; Figures 1C and 1D), confirming previous reports (Foster et al., 1996; Duffy et al., 2001; Artola et al., 2006; Li et al., 2006; Hu et al., 2010; Malik and Chattarji, 2012). EE significantly increased the number of $BrdU^+$ newborn neurons in the dentate gyrus by 289% (Figure S1D). Taken together, these various results indicate that our EE training protocol is effective in enhancing certain neural substrates of memory and learning in these wild-type mice, consistent with published EE studies.

EE Increases the Frequency of Miniature EPSCs and the Level of GluA1 pS845

To more fully characterize the neuronal effects of our EE paradigm, we performed patch-clamp whole-cell recordings that compared the frequency and amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in CA1 pyramidal neurons of SH and EE mice. Voltage-clamp recordings showed that the mEPSC frequency increased after EE (SH: 0.35 ± 0.05, events/sec, n = 14; EE: 0.56 ± 0.09, events/s, n = 14; $p < 0.01$), while mEPSC amplitude remained unchanged (SH: 26.23 ± 1.12 pA, n = 14; EE: 26.54 ± 0.70 pA, n = 14; $p > 0.05$; Figure 2A). These data suggested that EE increased neuronal excitation. To assess whether EE altered basal synaptic transmission, we measured input/output curves using extracellular field recordings and found no significant differences between SH and EE mice. (Figure S2A). Consistent with the data that EE increased spine volume and LTP, biochemical fractionation of hippocampus and quantitative WB revealed significant increases in the levels of certain postsynaptic receptors (Figure 2B) and signaling proteins (Figure S2B) in the EE mice. Although both LTP magnitude and mEPSC frequency were increased by EE, synaptic AMPAR subunit GluA1 levels did not change significantly. However, the phosphorylation of GluA1 at S845 (a PKA binding site) was significantly increased in EE versus SH mice (Figure 2B), consistent with previous reports that GluA1 phosphorylation correlates with lowered thresholds for LTP induction and memory formation (Hu et al., 2007). Thus, our EE paradigm leads to heightened expression of certain LTP-related signaling proteins and enhanced GluA1 phosphorylation.

EE Exposure Prevents Impairment of Hippocampal LTP by Aβ Oligomers

To learn whether this EE paradigm can ameliorate the adverse effects of Aβ oligomers on mechanisms of learning and memory, we studied the acute effects of soluble Aβ oligomers from several different sources on hippocampal LTP. Concentrations (~1–2 nM) of soluble human Aβ oligomers in the CM of 7PA2 cells (CHO cells stably expressing the hAPP-V717F AD mutant [Podlisny et al., 1995]) that fully inhibited LTP in hippocampal slices of SH mice still allowed a significant and normal LTP in EE slices (HFS: 108% ± 3%, n = 9/8, versus 145% ± 8%, n = 12/10; TBS: 113% ± 5%, n = 8/8, versus 144% ± 5%, n = 11/10; $p < 0.05$; Figures 3A and 3B). (The oligomer-rich 7PA2 CM had no significant effect on basal synaptic transmission in the hippocampal CA1 region in both SH and EE mice

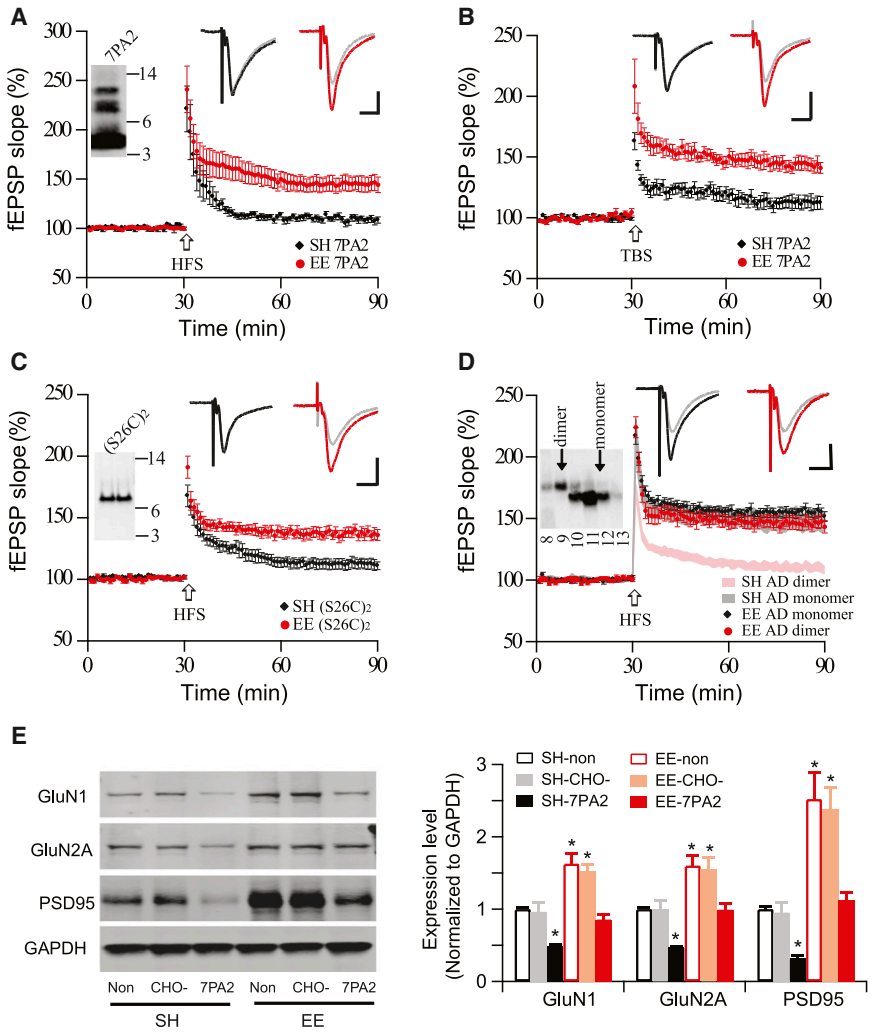

Figure 3. EE Exposure Prevents the Impairment of Hippocampal LTP and Decrease in Certain Signaling Molecules by Soluble Aβ Oligomers

(A) 7PA2 CM containing soluble human Aβ oligomers significantly inhibited HFS-induced LTP in hippocampal slices of SH mice (black diamonds, n = 9 slices/8 mice), but this was prevented in slices of EE-trained mice (red circles, n = 12/10). Inset: WB (Aβ mAbs 6E10+2G3) of the CM shows the soluble Aβ monomers (4 kDa) and SDS-stable low-n oligomers.

(B) 7PA2 CM significantly inhibited TBS-induced LTP in SH mice (black diamonds, n = 8/8) but not EE mice (red circles, n = 11/10).

(C) Synthetic human $A\beta_{1-40}$ S26C dimers (5 nM) significantly impaired hippocampal LTP in SH mice (black diamonds, n = 9/5) but not EE mice (red circles, n = 12/10). Inset: immunoblot of the $(S26C)_2$ used in the LTP recordings.

(D) IP-SEC fractionation of an AD TBS extract yielded dimer-rich SEC fraction #9 (inset) that inhibited HFS-induced LTP in SH mice (pink traces, n = 8/5), whereas monomer-rich fraction #12 (inset) did not (gray traces, n = 8/5). In EE mice, the dimer fraction did not inhibit LTP (red circles, n = 9/8), just like the monomer fraction (black diamonds, n = 8/8). Inset traces as in Figure 1.

(E) The decrease in synaptosomal proteins NMDAR and PSD95 induced by soluble Aβ oligomers is prevented by EE training. Left: representative WBs from hippocampal slices 6 hr after CHO- CM or 7PA2 CM treatment in SH and EE mice. Right: summary data from 4 mice in each group.

Error bars represent ±SEM (*p < 0.05; **p < 0.01). See also Figure S3.

[Figure S3A].) To confirm this benefit of EE, we tested two other sources of soluble Aβ oligomers: pure, synthetic $A\beta_{1-40}$S26C cysteine-crosslinked dimers (5 nM), and dimer-rich SEC fractions isolated from TBS-soluble cortical extracts of typical AD brains (Shankar et al., 2008; Li et al., 2009). The pure $(S26C)_2$ Aβ dimers markedly inhibited HFS-induced hippocampal LTP in SH mice, as expected (Li et al., 2011) (111% ± 4%, n = 9/5; Figure 3C). For EE mice, their enhanced LTP was reduced by the synthetic dimers, but this still left a substantial and significant LTP (138 ± 4%, n = 12/10) (Figure 3C). In the case of Aβ isolated directly from AD cortex, dimer-rich but not monomer-rich SEC fractions impaired LTP in SH mice (110% ± 3%, n = 8/5, versus 148% ± 8%, n = 8/5; $p < 0.001$) as reported (Li et al., 2011), and this LTP inhibition was almost fully prevented in the EE mice (dimers: 146% ± 5%, n = 9/8, versus monomers: 155% ± 4%, n = 8/8; $p > 0.05$; Figure 3D). Importantly, LTP in the presence of the AD brain dimers was statistically indistinguishable from that in AD brain monomers, which have consistently been shown to be electrophysiologically neutral (Shankar, et al., 2008; Li et al., 2009, 2011; Figure 3D). Interestingly, we found that EE training did not alleviate the known facilitation of hippocampal long-term depression (LTD) by soluble Aβ oligomers (Li et al., 2009) from all 3 Aβ sources tested (Figure S3B). Consistent with our electrophysiological and Golgi data, immunohistochemistry and quantitative WB both showed that EE training increased the area of synaptic puncta in the hippocampus (Figure S3C) and elevated LTP-relevant postsynaptic proteins (certain NMDA receptors and PSD95) (Figure 3E). In accord, the application of Aβ oligomer-rich 7PA2 CM to SH hippocampal slices lowered the numbers and areas of synaptic puncta (Figure S3C) as well as some LTP-related proteins (Figure 3E), but these decreases were restored to baseline levels in Aβ oligomer-treated slices of mice exposed to EE. Taken together, our results thus far suggest that EE training for 4 weeks early in life ameliorates the inhibition of hippocampal LTP by soluble Aβ oligomers, allowing a normal LTP and the restoration of synaptic structure.

EE-Enhanced LTP Requires Activation of β_2-Adrenergic Receptors

Previous studies described an increase in the contribution of cAMP signaling to LTP induction in the CA1 region of the hippocampus after 2–8 week exposures to EE (Duffy et al., 2001;

Li et al., 2006). Activation of cAMP/PKA signaling by the adenylyl cyclase activator, forskolin, or the phosphodiesterase type 4 (PDE4) inhibitor, rolipram, can restore the inhibition of LTP by Aβ in culture (Vitolo et al., 2002; Wang et al., 2009) and in APP tg mice (Gong et al., 2004). These reports could help explain how EE exposure prevented Aβ oligomer-impaired LTP in our mice. We found that two different PKA inhibitors, KT 5720 (5 μM) or H-89 (20 μM), reduced EE-enhanced LTP to its control (SH) levels (EE + KT: 140% ± 6%, n = 6/6, versus SH + KT: 138% ± 5% n = 6; EE + H89: 142% ± 5%, n = 8/6, versus SH + H89: 137% ± 4%; n = 7; Figure 4A). Conversely, activating PKA by pretreating slices with forskolin (20 μM) prevented the impairment of LTP by soluble Aβ oligomers in slices of SH mice (FSK + 7PA2 CM: 135% ± 4%, n = 8; 7PA2 CM alone: 108% ± 3%, n = 9; $p < 0.001$; Figure 4B), thereby mimicking the benefit of EE. To assess whether the prevention of Aβ oligomer-impaired LTP by EE shares similar mechanisms with activation of cAMP pathways, we applied forskolin (20 μM) to EE hippocampal slices 10 min prior to applying 7PA2 CM and found that the magnitude of LTP was not different from that with 7PA2 CM alone (Figure 4C). This occlusion experiment suggests that EE overcomes the detrimental effects of soluble Aβ oligomers in part via cAMP signaling pathways.

A key upstream effector of the PKA signaling pathway is the β-adrenergic receptor (β-AR). β-ARs are reported to undergo long-lasting modulation and increased activity after EE training (Escorihuela et al., 1995; Naka et al., 2002; Cao et al., 2010), but the electrophysiological effects have not been documented. To assess to what extent the enhancement of hippocampal LTP in our EE mice involved the activation of β-ARs, we applied the non-selective β-AR antagonist, propranolol (5 μM), to their hippocampal slices and found that LTP was normalized to the levels in SH mice (EE + prop: 146% ± 5%, n = 12/10, versus SH: 141% ± 4%, n = 7/7; $p > 0.05$; Figure 4D). Higher doses of propranolol dose-dependently inhibited LTP in both SH and EE hippocampal slices (Figure S4A). To characterize this role of β-ARs more precisely, we applied a weak HFS (which did not potentiate synaptic strength in slices of SH mice) to the slices of EE mice and observed a significant LTP (SH: 112% ± 3%, n = 14/12, versus EE: 138% ± 3%, n = 25/21; $p < 0.001$; Figure S4B). This LTP induced by weak HFS in the EE mice was fully blocked by either of two β-AR antagonists, propranolol (10 μM) (110% ± 4%, n = 10/8) or pronethalol (10 μM) (116% ± 5%, n = 7/7; Figure S4B), strongly implicating the activation of β-AR pathways in the effects of EE. In accord, treating slices from SH mice with the β-AR agonist, isoproterenol (iso), allowed a weak HFS to now induce a significant LTP (iso: 133% ± 4%, n = 8, versus ACSF: 114% ± 4% n = 8; $p < 0.01$; Figure S4C), consistent with a prior report (Gelinas et al., 2008). In agreement with this finding, pretreatment of SH slices with iso prevented the 7PA2 CM-induced inhibition of standard-HFS LTP (iso + 7PA2 CM: 143% ± 5%, n = 12/10; versus 7PA2 CM alone: 108% ± 3%, n = 10; $p < 0.001$; Figure 4E). Iso alone had a small and insignificant effect on LTP (Figure 4E) and had no effect on baseline fEPSPs over 2 hr (Figure S4D), longer than the time of LTP recording we used throughout the study. Aβ oligomers also did not affect baseline fEPSPs (Figure S4E). In an occlusion experiment, we applied iso to EE hippocampal slices 10 min prior to applying 7PA2 CM and found that the magnitude of LTP was the same as with 7PA2 CM alone, again suggesting that iso and Aβ oligomers share mechanistic pathways (Figure 4C). Iso also fully prevented the inhibition of HFS-induced LTP by synthetic S26C dimers in SH slices (dimer + iso: 147% ± 7%, n = 7; dimer alone: 112% ± 4%, n = 7; $p < 0.001$; Figure S4F). Intriguingly, if we treated slices with Aβ oligomers 10 min *before* the application of iso, there was no restoration of LTP (Figure S4F, blue triangles). Alternative upstream modulators of cAMP signaling, namely dopamine agonists (chloro-APB HBr [5 μM] or SKF 38393 [50 μM]), did not enable weak HFS to induce a significant LTP in SH slices, in contrast to iso (Figure S4C). In a pharmacologically specific experiment, the selective β_2-AR antagonist, ICI 118551 (100 nM), but not the selective β_1-AR antagonist, metoprolol (10 μM), fully blocked the weak HFS-induced LTP in EE mice (ICI: 107% ± 3%, n = 7; metoprolol: 132% ± 5%, n = 8; $p < 0.001$; Figure 4). Consistent with these electrophysiological findings, immunoblotting of hippocampal synaptosomes from EE versus SH mice detected a significantly increased expression of β_2-AR with EE, but not β_1-AR, and dopamine receptors (Figures 4G and 4H) and serotonin and muscarinic acetylcholine receptors (Figure S4G). Taken together, these various results indicate that activation of the β_2-AR signaling pathway in particular can prevent soluble Aβ oligomers from impairing hippocampal LTP in wild-type mice.

Aβ Oligomers Inhibit and Induce Internalization of β_2-Adrenergic Receptors

Previous reports showed decreases in the levels of β-ARs and norepinephrine in several regions of AD brain (Marien et al., 2004; Szot et al., 2006; Manaye et al., 2013), and Aβ-may induce internalization of β-ARs (Wang et al., 2011). We next examined the expression of β_2-AR and other synaptic proteins in hippocampal slices after treatment with soluble Aβ oligomers for increasing times. The phosphorylation of β_2-AR at Thr 384, which is phoshorylated by GRK2 (Fredericks et al., 1996) and responsible for β2-arrestin-dependent receptor desensitization (Gurevich and Gurevich, 2006), was increased and the total expression of β_2-AR was decreased in the synaptosomes of hippocampal slices just 1 hr after administration of 7PA2 CM, whereas NMDAR and PSD95 were not significantly changed at this early time (Figure 5A). After 6 hr treatment, the levels of β_2-ARs, NMDARs and PSD95 were all decreased significantly by the 7PA2 CM but not the control CHO- CM (Figure 5A), while phosphorylation of β_2-AR at Thr384 had returned to normal. Moreover, surface biotinylation revealed that the cell-derived soluble Aβ oligomers enhanced internalization of β_2-AR (but not β_1-AR, dopamine, muscarinic acetylcholine or serotonin receptors) in cultured rat hippocampal neurons (Figures 5B and S5). Overall, these results suggest that the activation of β2-AR by EE can restore impairments of selected synaptic proteins by soluble Aβ oligomers.

Prolonged Feeding of a β-Adrenergic Receptor Agonist Mimics EE Effects In Vivo

In view of the striking amelioration of Aβ-mediated effects on synaptic plasticity by β-AR manipulation in acute slices, we extended this approach to the in vivo condition. We added the

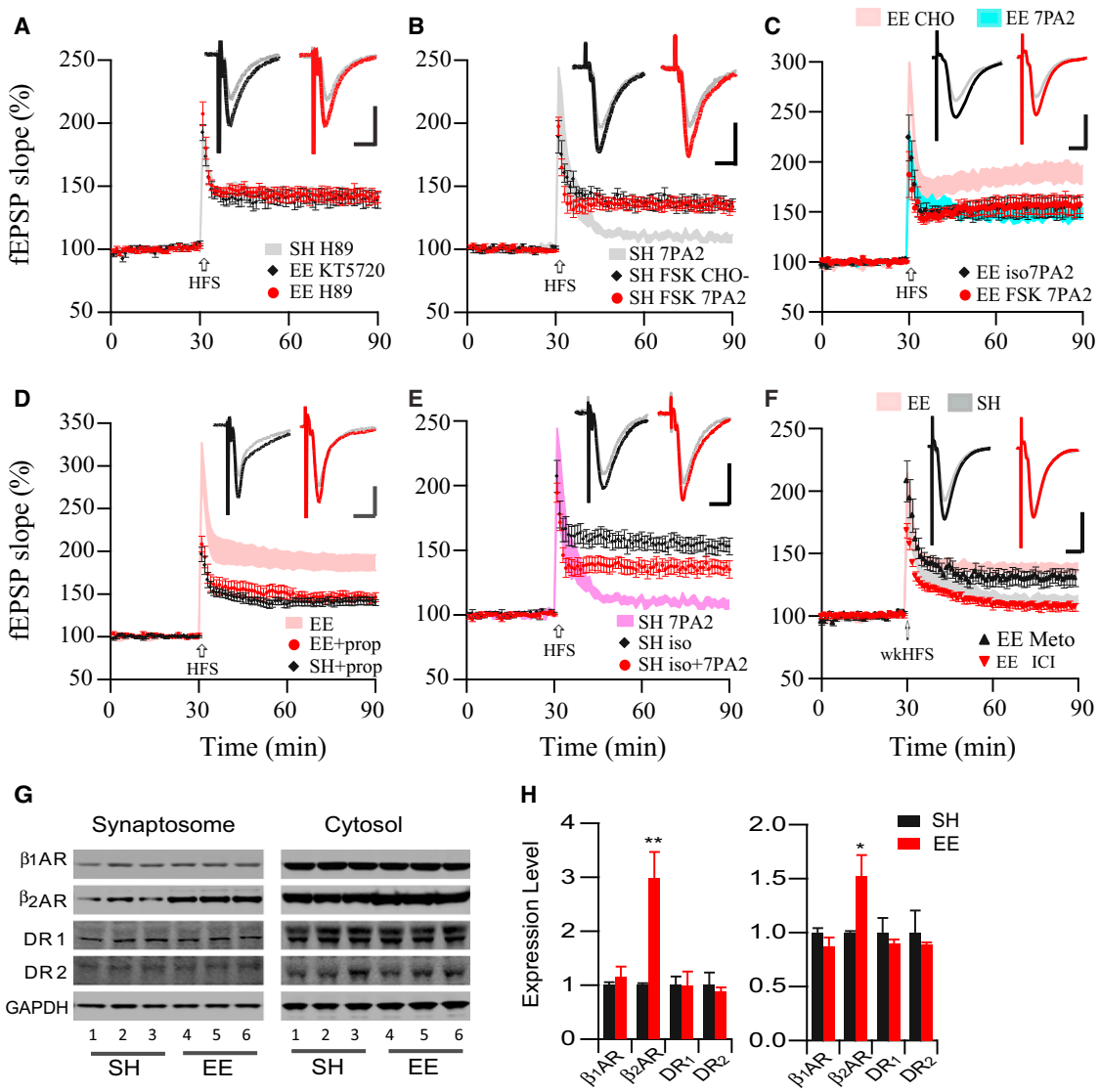

Figure 4. EE-Enhanced LTP Requires Activation of β-Adrenergic Receptors in the CA1 Region of Hippocampus

(A) The enhancement of LTP in EE slices was blocked by applying PKA inhibitors KT5720 (5 μM; black diamonds, n = 6/6) or H-89 (20 μM, red circles, n = 8/6), bringing the LTP down to levels in SH slices (gray trace).

(B) Activating cAMP signaling with forskolin (FSK, 20 μM) prevented the Aβ oligomers in 7PA2 CM from impairing LTP (red circles, n = 8) in SH slices. Gray trace: SH slices without forskolin pretreatment show the LTP blocked by 7PA2 CM. FSK alone (black diamonds) had no effect on LTP.

(C) Pretreatment with isoproterenol (iso, 10 μM, black diamonds, n = 6) or forskolin (FSK, 20 μM, red circles, n = 6) in hippocampal slices from EE mice does not further affect the magnitude of HFS-induced LTP in the presence of 7PA2 CM. Pink trace: CHO- CM alone in EE slices; light blue trace: 7PA2 CM alone in EE slices.

(D) Treating EE slices with the β-AR antagonist propranolol (prop, 5 μM) decreased the EE-enhanced LTP (red circles, n = 12/10) down to the level of SH slices (black diamonds, n = 7/7). Pink trace: EE slices alone.

(E) The β-AR agonist isoproterenol (iso, 10 μM) prevented the inhibition of LTP by 7PA2 CM in SH slices (red circles, n = 12/10), mimicking the effect of EE. Iso itself did not significantly enhance LTP (black diamonds, n = 13/8). Pink trace: 7PA2 CM alone.

(F) EE-facilitated hippocampal LTP induced by a weak HFS is blocked by the selective β_2-AR antagonist ICI 118551 (100 nM, n = 6/8, red triangles), but not by β_1-AR antagonist, metoprolol (10 μM, n = 6/7, black triangles). The weak HFS induced a significant LTP in slices of EE mice (pink trace), while it failed to induce LTP in slices of SH mice (gray trace).

(G) Immunoblots of monoamine receptor levels after 4 wk EE training versus in SH. Hippocampal synaptosome and cytosol fractions of fresh brain tissue were probed with antibodies to β_1- or β_2-adrenergic receptors or dopamine D1 or D2 receptors. GAPDH, loading control.

(H) Summary data from WB exemplified in (G); expression levels are normalized to GAPDH. Left graph, hippocampal synaptosomes; right graph, hippocampal cytosol.

Error bars represent ±SEM (*p < 0.05; **p < 0.01). Inset traces in (A)–(F) as in Figure 1. See also Figure S4.

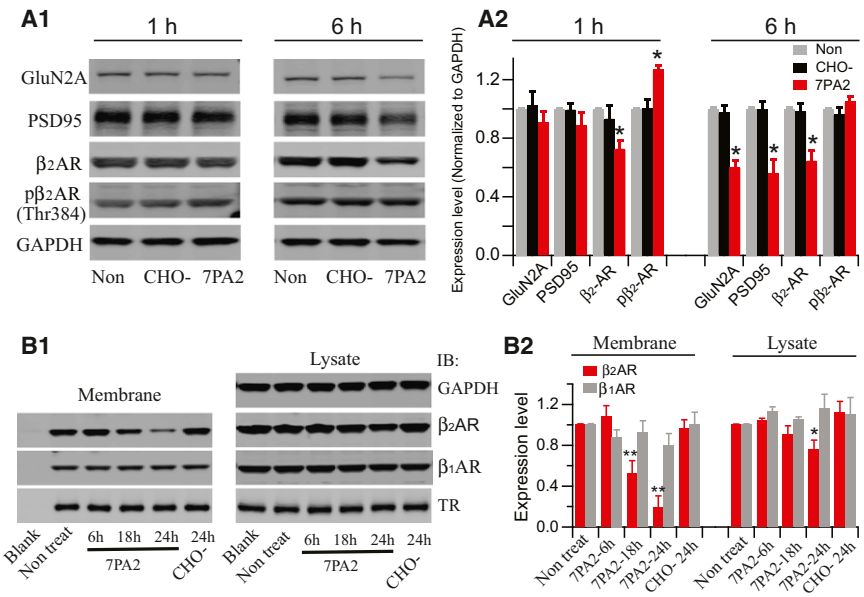

Figure 5. Aβ Oligomers Decrease Levels and Induce Internalization of β_2-Adrenergic Receptors

(A) Levels of β-AR and other synaptic proteins in the synaptosomes of hippocampal slices after treatment with Aβ oligomers for increasing times. (A1) Representative WB of protein levels in hippocampal synaptosomes. (A2) Summary data (bars) (*$p < 0.05$).

(B) Soluble Aβ from 7PA2 CM induced the selective internalization of β_2-ARs biotinylated at the cell surface (membrane), but not that of β_1-ARs or dopamine receptors 1 or 2, in primary rat hippocampal neurons. Note the time-dependent decrease of surface β2-AR levels at 6, 18, and 24 hr exposure to 7PA2 CM versus no effect with 24 hr of CHO- CM. TR, biotinylated surface transferrin receptor as a loading control. (B1) Representative WB of protein levels. (B2) Summary data (bars). Protein levels are normalized to TR for membrane fraction and to GAPDH for lysate.

Error bars are ±SEM (*$p < 0.05$; **$p < 0.01$). See also Figure S5.

β-AR antagonist, propranolol, to the drinking water (at 0.2 g/l, as per Cao et al., 2010) of 2-week-old mice undergoing the 4 week EE training. The usual enhancement of LTP by the 4 weeks of EE was now reduced to SH levels (EE + prop: 144% ± 4%, n = 13/13 versus SH: 146% ± 5%, n = 9/5, $p > 0.05$; Figure 6A). In accord, EE in these propranolol-fed mice failed to prevent the impairment of LTP by soluble Aβ oligomers, while EE mice who consumed just water resisted the Aβ synaptotoxicity, as before (prop: 114% ± 3%, n = 14/12; water: 149% ± 6%, n = 14/14; $p < 0.001$; Figure 6B). Conversely, SH mice receiving the β-AR agonist isoproterenol (0.1 g/l) in their drinking water for 4 weeks developed a significant resistance to the LTP-inhibiting effect of the Aβ oligomers (iso: 137% ± 4%, n = 9/8; water: 113% ± 3%, n = 10/6; $p < 0.001$; Figure 6C). Similarly, the iso-fed SH mice showed normal LTP in slices treated with pure S26C Aβ dimers (iso: 141% ± 5%, n = 7; water: 118% ± 3%, n = 7; $p < 0.001$; Figure 6D). To verify these electrophysiological findings, we also performed quantitative WB on the EE mice fed propranolol and the SH mice fed isoproterenol. Consistent with the LTP findings, the beneficial biochemical effects of EE could be significantly decreased by feeding the β-AR antagonist propranolol, and they could be mimicked by feeding the β-AR agonist, isoproterenol, to SH mice (Figures 6E and S6).

Novelty Is More Effective Than Exercise in Preventing Aβ-Induced LTP Inhibition

We next asked whether physical exercise or novelty played a greater role in the Aβ-protective effects of our EE paradigm. We separated these factors by exposing mice either principally to physical exercise in large cages containing just two running wheels (RW group) or principally to cognitive enhancement by exploring multiple, changing novel objects in large cages without running wheels (novelty [Nov] group). Intriguingly, the novelty group developed significantly increased hippocampal LTP similar to the levels in our earlier combined EE group, whereas LTP in the RW group was not significantly different from that of SH mice (Nov: 180% ± 10%, n = 16/13; RW: 158% ± 6%, n = 13/11; $p = 0.049$; Figure 7A). In accord, exposure to novelty, but not to the RW, overcame the inhibition of LTP by cell-derived human Aβ oligomers (Nov: 141% ± 5%, n = 9/9; RW: 119% ± 4%, n = 9/7; $p < 0.01$; Figure 7B). Similar results were seen upon treatment of hippocampal slices from the respective groups with pure synthetic S26C dimers (Nov: 144% ± 6%, n = 8/6; RW: 127% ± 6%, n = 8/6; versus SH: 112% ± 3%, n = 8; $p < 0.05$; Figure 7C); here the RW group showed mild protection against the Aβ-mediated inhibition of LTP. These findings suggest that prolonged and repetitive exploration of a complex novel environment may beneficially modulate hippocampal synaptic plasticity and reduce synaptotoxic effects of Aβ oligomers in ways that physical exercise alone does not provide.

Prolonged Exposure to EE in Midlife Protects against Aβ Oligomers

To make our findings more relevant to how prolonged exposure to an enriched cognitive environment might benefit adults with a propensity to Aβ accumulation and eventual development of AD, we sought to determine whether EE initiated in mature adult mice could achieve analogous benefits. We began the same EE training paradigm used at age 2–6 weeks (above) in mice aged 5 months. A 4 week EE exposure slightly enhanced hippocampal LTP, but this did not achieve statistical significance (EE: 177% ± 16%, n = 11/9; SH: 153% ± 8%, n = 8/6; $p > 0.05$; Figure 7D). While a 4 week EE exposure in adults thus had less robust effects on the magnitude of LTP than in young animals, the partial resistance of LTP to the inhibitory effects of soluble Aβ oligomers did achieve significance (EE + 7PA2: 128% ± 6%, n = 11/10; SH + 7PA2: 114% ± 4%, n = 9/6: $p < 0.05$; Figure 7D). To extend this approach, we next exposed 5-month-old adult mice to an EE training period of 8 weeks. Now, EE exposure produced similar benefits to those observed in young mice trained for 4 weeks: LTP in the 8 week EE mice (i.e., at age 7 months) was 195% ± 9% (n = 20/16) versus 153% ± 7% (n = 11/8) in

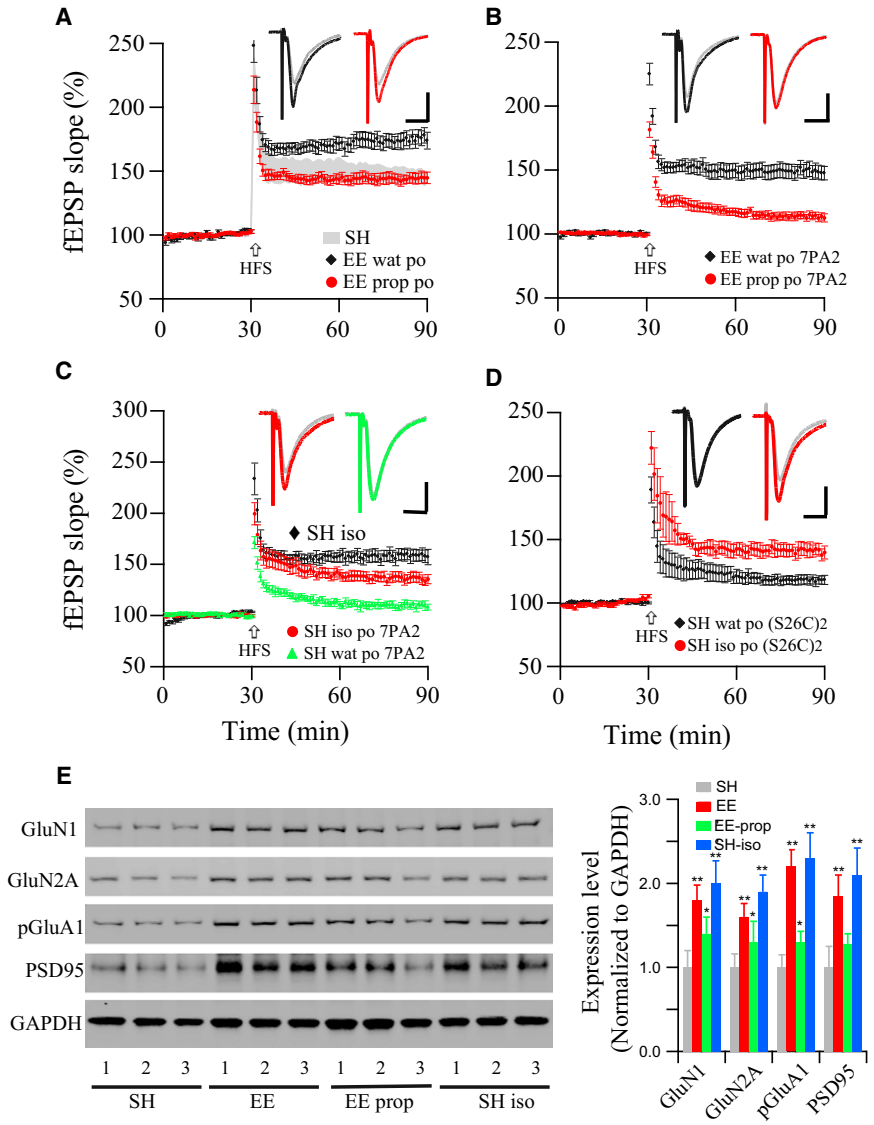

Figure 6. EE-Enhanced LTP Can Be Mimicked by Prolonged Oral Administration of a β-Adrenergic Receptor Agonist

(A) Prolonged oral administration of propranolol (prop, 0.2 g/l) in drinking water during EE training significantly decreased the EE-enhanced hippocampal LTP (red circles, n = 13/13) to levels in SH mice (gray trace), while EE mice on plain water (wat) showed the usual LTP enhancement (black diamonds, n = 9/5).

(B) 7PA2 CM markedly inhibited LTP in slices of EE mice given propranolol in the drinking water (red circles, n = 14/12), versus the normal LTP in EE mice on plain water (wat) (black diamonds, n = 14/14).

(C) Oral administration of isoproterenol (iso, 0.1 g/l) for 4 wk to SH mice mimicked the EE effect on LTP (black diamonds, n = 10/7), and iso prevented the block of LTP by 7PA2 CM (red circles, n = 9/8). The same 7PA2 CM blocked LTP in SH mice on plain water (green triangles, n = 10/6).

(D) Giving SH mice iso (0.1 g/l) in drinking water for 4 wk fully prevented the block of LTP by pure Aβ $(S26C)_2$ dimers (5 nM) (red circles, n = 7/7). The same dimers significantly blocked LTP in SH mice on plain water (black diamonds, n = 7).

(E) WB of selected signaling proteins in hippocampal slices of untreated SH and EE mice, EE mice fed propranolol, and SH mice fed isoproterenol. Representative WB show the protein levels from hippocampal synaptosomes. Summary quantification shown in bar graphs.

Error bars represent ±SEM (*p < 0.05; **p < 0.01). Inset traces as in Figure 1. See also Figure S6.

SH mice (p < 0.001) (Figure 7E). Moreover, the Aβ oligomer-mediated inhibition of LTP was fully prevented (EE + 7PA2 CM: 156% ± 6% [n = 12/12] versus SH + 7PA2 CM: 115% ± 4% [n = 11/6]; p < 0.001; EE + $(S26C)_2$: 144% ± 5% (n = 14/10) versus SH + $(S26C)_2$: 118% ± 4% (n = 10/8); p < 0.001; Figures 7E and 7F). We conclude that prolonged environmental enrichment initiated in adult wt mice significantly enhances hippocampal synaptic plasticity, providing resistance to the adverse synaptic effects of soluble Aβ oligomers on the hippocampus.

DISCUSSION

With the rising prevalence of AD and the challenge of developing disease-modifying drugs that can be taken safely for decades, nonpharmacological approaches to delay the onset of AD or ameliorate its course deserve intensive study. In mice overexpressing mutant human APP and having robust Aβ deposition, a number of reports suggest that exercise and/or EE can decrease AD-like pathology and lessen behavioral deficits, although there is disagreement about the effects on plaque burden and incomplete knowledge of the responsible mechanisms (see Table S1). Many late-onset AD cases occur insidiously without known disease-promoting genetic precipitants, so that a complex mix of environmental and genetic factors is likely to contribute to their pathogenesis. We are unaware of prior studies of the potential benefits of EE in wild-type animals with no genetic diathesis for Aβ deposition. There are also no reports of the effects of EE on the synaptotoxicity of soluble Aβ oligomers per se, yet these species are believed to be the earliest and most bioactive form of β-amyloid. By applying soluble oligomers—including those isolated directly from AD brains—to the hippocampus of wt mice, we distinguish the effects of EE on synaptic function versus on Aβ neuropathology. We demonstrate that EE, and novelty in particular, potently protect against synaptic impairment by human Aβ oligomers, in part by activating a β_2-adrenoreceptor signaling pathway that can also be beneficially activated by prolonged oral administration of a β-AR agonist.

The noradrenergic transmission system is known to participate in various forms of learning and memory. Activation of β-ARs was shown to be required for the enhancement of

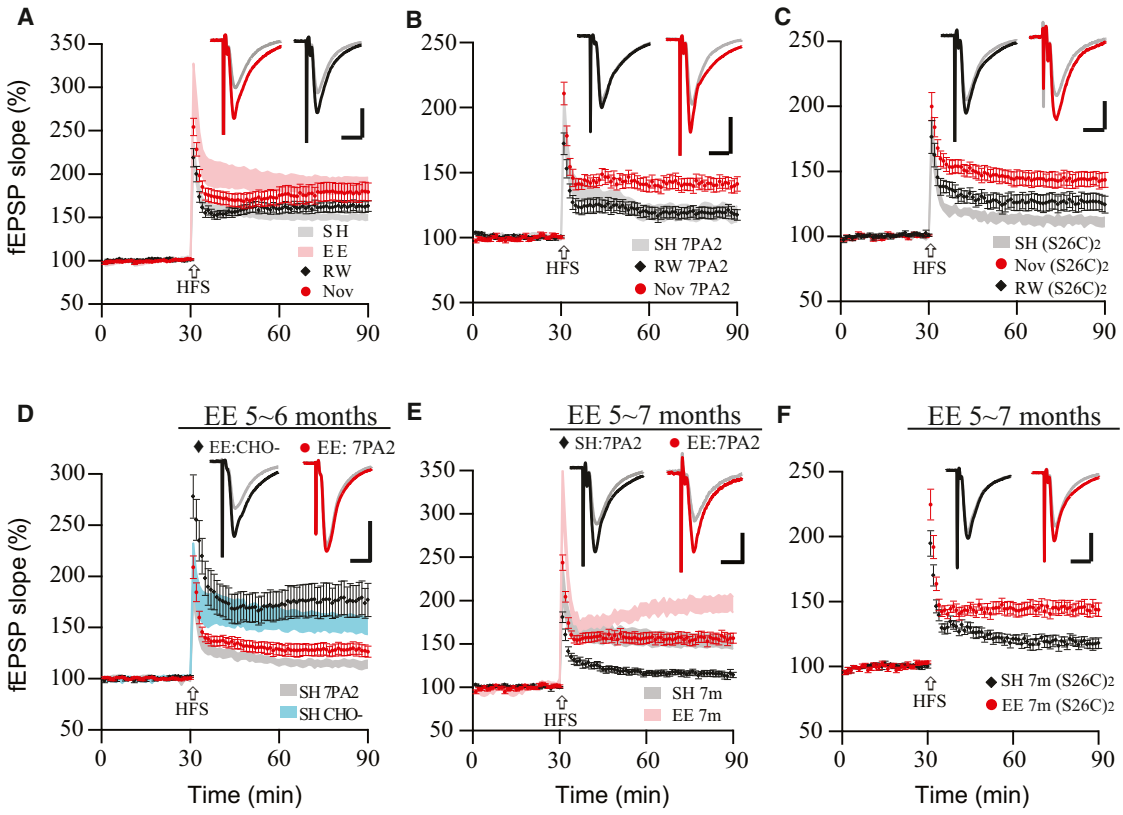

Figure 7. Novelty Exposure Prevents Aβ Oligomer-Induced LTP Inhibition More Effectively Than Does Exercise, and Midlife EE Is Also Beneficial

(A) Prolonged exposure to novelty (Nov) (red circles, n = 16/13) significantly enhanced hippocampal LTP to the level of our usual EE paradigm (pink trace), whereas exposure to just running wheels (RW) (black diamonds, n = 13/11) did not significantly enhance LTP above the level in SH mice (gray trace).

(B) Exposure to novelty (red circles, n = 9/9) but not physical exercise (black diamonds, n = 9/7) significantly prevented LTP inhibition by the Aβ oligomer-rich 7PA2 CM.

(C) Novelty (red circles, n = 8/6) fully prevented LTP inhibition by Aβ $(S26C)_2$, and physical exercise (RW) did so significantly but less (black diamonds, n = 8/6).

(D) Standard EE training of adult mice from age 5 to 6 months enhanced LTP (black diamonds, n = 11/9) but less than occurred in young mice (c.f. Figures 1C and 1D). This 4 week EE produced modest but significant protection against 7PA2 Aβ oligomers (red circles, n = 11/10).

(E) Eight weeks EE in mature adult mice from 5 to 7 months had similar benefit as 4 week EE in young mice, yielding significant LTP enhancement (pink trace) and preventing the block of LTP by Aβ oligomers (red circles, n = 12/12). As expected, the oligomers strongly inhibited LTP in SH mice at 7 months (black diamonds, n = 11/6).

(F) EE in adults (5–7 months) fully prevented LTP impairment by Aβ $(S26C)_2$ (red circles, n = 14/10) versus the block seen in SH mice (black diamonds, n = 10/8). Data are means ± SEM. Insets as in Figure 1.

hippocampal LTP by repetitive exploration of a novel environment (Straube et al., 2003; Kemp and Manahan-Vaughan, 2008; O'Dell et al., 2010). β-AR activation is strongly implicated in memory storage (McGaugh et al., 1996) and in the benefits of novelty exposure (King and Williams, 2009; Lemon et al., 2009). Conversely, β-AR blockade can impair attention, learning and memory in animals and humans (Cahill et al., 2000; Chamberlain et al., 2006). In AD brain tissue, decreases in the levels of β-ARs and norepinephrine have been reported in several brain regions (Marien et al., 2004; Szot et al., 2006; Manaye et al., 2013), presumably due in part to the observed loss of noradrenergic neurons in the locus coeruleus (Grudzien et al., 2007; Weinshenker, 2008; Manaye et al., 2013). One possible mechanism for the reduced β-AR levels in AD is an Aβ-induced internalization of the receptors (Wang et al., 2011), and this is just what we find after the application of soluble human Aβ oligomers (Figure 5B). Activation of the β_2AR and its downstream cAMP/PKA signaling pathways can prevent Aβ-mediated inhibition of LTP (Wang et al., 2009). These findings are all consistent with evidence that pharmacological enhancement of cAMP/PKA signaling restores Aβ-impaired LTP in both wild-type (Vitolo et al., 2002) and APP transgenic (Gong et al., 2004) mice. EE can increase norepinephrine concentrations and strengthen the β-AR signaling pathways in the brain (Escorihuela et al., 1995; Naka et al., 2002). All of these data are consistent with our finding that EE potently protects against Aβ oligomer-mediated synaptic dysfunction. It will now be important to conduct detailed comparisons of the effects of novel environments and β-AR stimulation in wt versus APP tg mice, in order to ascertain the respective effects of EE on Aβ homeostasis versus synaptic structure and function.

In this study, mice exposed to novelty alone showed more electrophysiological benefit than those solely offered running wheels. This finding may relate to our observation that the mice moved rapidly among the novel objects they were exploring, providing two salutary factors (cognitive exploration and physical activity). Novelty exploration causes an increase in activity of the locus coeruleus and increases hippocampal norepinephrine release (Sara et al., 1994). It also increases several physiological indices of arousal, including heart rate and blood pressure, as physical exercise does via activation of the sympathetic nervous system. Our results are consistent with a report that physical exercise alone was less effective than novelty training in protecting against cognitive deficits in APP transgenic mice (Cracchiolo et al., 2007).

Epidemiological studies in humans suggest that environmental influences, including cognitive activity (childhood intelligence, higher education, job complexity), social interactions, and physical exercise, may all delay the onset of dementia in AD (Paradise et al., 2009). Our controlled experimental study of EE in mice at two ages suggests that increased cerebral activity for 4 weeks early in life contributes a strong protective effect against Aβ oligomer-mediated synaptotoxicity. However, longer (8 week) EE exposure in our adult animals still provided clear-cut benefits in terms of resistance to the toxicity of soluble Aβ oligomers. Our EE protocols are analogous to prolonged cognitive and physical activity applied before the onset of significant Aβ accumulation and the consequent development of cognitive symptoms. Although many humans may miss opportunities for heightening of cognitive activities earlier in life, our results in healthy adult mice suggest that benefits can accrue from novelty exposure in middle age if it is more prolonged and intensive. These observations are consistent with certain epidemiological studies of mid- and late-life exposure to enriched or complex environments that have shown measurable beneficial effects on cognition and on the risk for AD dementia (Wang et al., 2002; Verghese et al., 2006). Increased physical activity has also been shown to protect against late-life cognitive decline and dementia in several studies (e.g., Lautenschlager et al., 2008; Baker et al., 2010). Our mechanistic data on EE, particularly novelty, and β-adrenergic stimulation suggest that it may be possible to ameliorate the early pathophysiological effects of soluble Aβ oligomers in humans by manipulating the environment. Thus, prolonged presymptomatic behavioral modification involving cognitive novelty and exercise could complement Aβ-directed and other preventative pharmacological agents to delay the onset of AD.

EXPERIMENTAL PROCEDURES

Animals

The Harvard Medical School Standard Committee on Animals approved all experiments involving mice used for electrophysiology and biochemical assays. All mice (male and female) contained a mixed background of C57Bl/6 and 129. Animals were housed in a temperature-controlled room on a 12 hr light/12 hr dark cycle and had ad libitum access to food and water.

Environmental Enrichment

Mice at age 14 days were randomly divided into groups of 4–6 placed into either standard housing (SH) or an enriched environment (EE). SH is a common housing cage (25 × 20 × 15 cm). EE cages are larger (60 × 38 × 20 cm) and contain 2 running wheels and multiple plastic toys/objects of varying shapes and colors. The toys were changed daily. Mice were housed in SH or EE for 4 weeks (2 to 6 weeks of age). In some experiments, 5-month-old adult mice were exposed to EE for either 4 or 8 weeks. The activity of the individual mice (exploring the objects and/or running on a wheel) was monitored 3–5 times during each 8 hr EE training session; mice showing little voluntary activity received a tail mark, and a very few animals that persistently showed no interest in exploratory or running activity were removed from the EE groups. Control littermate mice were housed in the same room in standard cages (15 × 25 cm) with only bedding and access to water and food pellets (SH).

Cellular Aβ Preparations

Secreted human Aβ peptides were collected and prepared from the conditioned media (CM) of a CHO cell line (7PA2) that stably expresses human APP751 containing the V717F AD mutation (Podlisny et al., 1995). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, and 200 mg/ml G418 for selection. Upon reaching ~95% confluency, the cells were washed and cultured overnight (~15 hr) in serum-free medium. CM was collected, spun at 1,500 × g to remove dead cells and debris, and stored at 4°C. The CM was concentrated 10-fold with a YM-3 Centricon filter (Walsh et al., 2005). Aliquots of concentrated 7PA2 CM were stored at –80°C. Disulfide crosslinked dimers of a human Aβ1-40 S26C synthetic peptide (gift of D. Walsh, BWH/HMS) were purified by size-exclusion chromatography.

Immunoprecipitation and Size Exclusion Chromatography

Immunoprecipitation and size exclusion chromatography are described in Supplemental Experimental Procedures.

Hippocampal Slice Preparation

Mice (C57BL/6 × 129) in either SH or EE cages were euthanized with Isoflurane at 6 weeks or at 6 or 7 months of age. Brains was quickly removed and submerged in ice-cold oxygenated sucrose-replaced artificial cerebrospinal fluid (ACSF) cutting solution (206 mM sucrose, 2 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose [pH 7.4], 315 mOsm. Transverse slices (350 μm thickness) from the middle portion of each hippocampus were cut with a vibroslicer. After dissection, slices were incubated in ACSF that contained the following (in mM): 124 NaCl, 2 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose [pH 7.4], 310 mOsm, in which they were allowed to recover for at least 90 min before recording. A single slice was then transferred to the recording chamber and submerged beneath continuously perfusing ACSF that had been saturated with 95% O_2 and 5% CO_2. Slices were incubated in the recording chamber for 20 min before stimulation under room temperature (~26°C).

Electrophysiological Recordings

We used standard procedures to record field excitatory postsynaptic potentials (fEPSP) in the CA1 region of the hippocampus. A bipolar stimulating electrode (FHC Inc., Bowdoin, ME) was placed in the Schaffer collaterals to deliver test and conditioning stimuli. A borosilicate glass recording electrode filled with ACSF was positioned in stratum radiatum of CA1, 200~300 μm from the stimulating electrode. fEPSP in the CA1 region were induced by test stimuli at 0.05 Hz with an intensity that elicited a fEPSP amplitude 40%–50% of maximum. Test responses were recorded for 30–60 min prior to beginning the experiment to assure stability of the response. Once a stable test response was attained, experimental treatments (Aβ oligomers, and/or other compounds) were added to the 10 ml ACSF perfusate, and a baseline was recorded for an additional 30 min. To induce LTP, two consecutive trains (1 s) of stimuli at 100 Hz separated by 20 s were applied to the slices, a protocol that induced LTP lasting approximately 1.5 hr in wild-type mice of this genetic background. To induce LTD, 300 or 900 pulses were delivered at 1 Hz. The field potentials were amplified 100× using an Axon Instruments 200B amplifier and digitized with Digidata 1322A. Data were sampled at 10 kHz and filtered at 2 kHz. Traces were obtained by pClamp 9.2 and analyzed using the Clampfit 9.2 program. LTP and LTD values reported throughout were measured at 60 min after the conditioning stimulus unless stated otherwise. Paired-pulse

responses were monitored at 50 ms interstimulus intervals. The facilitation ratio was calculated as fEPSP2 slope/fEPSP1 slope. Two-tailed Student's t test and one-way analysis of variance (ANOVA) were used to determine statistical significance.

Whole-Cell Recordings

Whole-cell recordings were made from the soma of visually identified pyramidal neurons located in CA1 of the hippocampus. Patch pipettes (5–7 MΩ) were filled with an internal solution containing (in mM): 110 Cs-gluconate, 20 CsCl, 10 HEPES, 4 NaCl, 0.5 EGTA, 2 $MgCl_2$, 2 Na_2ATP, and 0.25 NaGTP, titrated with KOH to pH 7.4, 290–300 mOsm. Series resistance was kept 15–30 MΩ and was monitored throughout each recording. Cells were excluded from data analysis if the series resistance changed by >20% during the course of the experiment. AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of −70 mV in the presence of 10 μM bicuculline methiodide (BIC) and TTX (0.5 μM). Continuous current traces of 5 min duration (recorded at least 10 min after achieving whole-cell configuration) were analyzed. All patch clamp experiments were performed at room temperature (24°C).

Golgi Staining

Golgi staining is described in Supplemental Experimental Procedures.

Preparation of Synaptosome and Cytosolic Fractions and Quantitative Western Blotting

Synaptosome and cytosolic fractions were prepared as described before (Li et al., 2011) with minor modifications. Hippocampi and motor cortex were dissected from mice under different conditions of treatment (n = 6 for SH and EE), and then the tissues were sectioned by vibratome at 350 μm thickness and collected in ice-cold sucrose homogenization buffer (40 μl per 10 mg tissue) containing the following (in mM): 320 sucrose, 10 Tris (pH 7.4), 1 Na_3VO_4, 5 NaF, 1 EDTA, and 1 EGTA. Slices were then homogenized in a glass grinding vessel using a rotating Teflon pestle (2,000 rpm) with at least 20 passes to create a Dounce homogenate. The homogenate was centrifuged at 1,000 × g for 10 min to remove nuclei and incompletely homogenized material (P1). The resulting supernatant (S1) was spun at 10,000 × g for 15 min to obtain a P2. The supernatant (S2) was defined as cytosolic fraction. The P2 was subsequently resuspended in 120 μl sucrose buffer using a motorized pestle mixing/grinding rod (Kontes) directly in the microfuge tube with 30 pulses. The P2 was then subjected to detergent extraction by adding 8 vol of Triton X-100 buffer (final = 0.5% v/v) containing the following reagents (in mM): 10 Tris (pH 7.4), 1 Na_3VO_4, 5 NaF, 1 EDTA, and 1 EGTA. This suspension was incubated at 4°C for 20 min with gentle. We operationally defined the suspension as the synaptosome fraction. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. We loaded 20 μg of total protein in each lane, separated by 4%–12% SDS-PAGE and blotted onto nitrocellulose membrane. The blot was blocked for 1 hr at RT, followed by incubation overnight at 4°C with rabbit polyclonal antibodies: GluN1 (Millipore), GluN2A (Millipore), GluN2B (Invitrogen), GluA1 (Millipore), GluA1 pS845 (Thermo), Synapsin I (Millipore), p-CREB (Millipore), p-ERK1/2 (Cell Signaling), p-CaMKII (Millipore), β2-adrenergic receptor (Thermo), β1-adrenergic receptor (Thermo), β2-adrenergic receptor Thr384 (Millipore), Dopamine Receptor 1 (Millipore), Dopamine Receptor 2 (Millipore), AchR M1 (Millipore), AchR M2 (Millipore), 5HTR 1B (Millipore), 5HTR 2B (Millipore), or mouse monoclonal antibodies: PSD95 (Millipore), Synaptophysin (Millipore), GAPDH (Millipore). Membranes were rinsed and incubated for 1 hr with fluorescence-conjugated goat anti-rabbit or mouse IgG (1:5,000; Invitorgen). Blots were scanned using a Li-Cor Odyssey system.

Immunohistochemistry and Confocal Microscopy

Immunohistochemistry and confocal microscopy are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.12.040.

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